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A cold staining method for acid-fast bacilli

R. VASANTHAKUMARI,[1] K. JAGANNATH,[2] & S. RAJASEKARAN[3]

The Ziehl-Neelsen method is probably the best known and most frequently used procedure for staining tubercle bacilli. The method requires controlled heating for its success. However, in developing countries, such as India, where most laboratories rely mainly on spirit lamps as a source of heat, the Ziehl-Neelsen method often cannot be carried out because rectified spirit is difficult to obtain. The study describes a cold staining technique that uses the same staining solutions as the conventional Ziehl-Neelsen method. For direct smears, the correlation of results of the cold staining procedure with those of the Ziehl-Neelsen method was 97% and for concentrated smears was 99%. The method described is suitable for use in basically equipped laboratories.

The Ziehl-Neelsen method is the most common laboratory technique for staining acid-fast tubercle bacilli. The surface of these bacteria contains a large amount of unsaponifiable waxy substance, and a number of staining methods have been developed to increase the permeability of the cell wall to the stain used. In the Ziehl-Neelsen method, the basic fuchsin-phenol dye is used hot, thus melting the waxy substance and increasing the permeability of the cell wall. However, because of certain disadvantages of this technique, there have been various attempts to develop a cold method for staining tubercle bacilli. For example, Kinyoun used higher concentrations of basic fuchsin and phenol (*1*), while Tax Thiam Hok devised a method (*2*) by combining the staining techniques of Kinyoun and Gabbet (*3*), alleging that it was superior to and quicker than the Ziehl-Neelsen method. In contrast, Rao et al. used chloroform instead of ethanol as solvent, without increasing the concentration of the basic fuchsin-phenol staining solution, and reported that this variation was as efficient as the Ziehl-Neelsen method (*4*).

We report here a variation of the Ziehl-Neelsen method that permits the cold staining of tubercle bacilli. The procedure uses the same reagents as the conventional Ziehl-Neelsen method, but avoids the need to use heat by increasing the staining time.

MATERIALS AND METHODS

Sputum samples from patients with pulmonary tuberculosis who had been admitted to the Government Tuberculosis Sanatorium, Tambaram, India, were used in the study. Smears prepared from the sputa were stained using both the conventional Ziehl-Neelsen method and the cold staining method.

Altogether 1100 samples of sputum were collected and divided into two groups, consisting of 700 and 400 samples, respectively. For the larger of these groups, direct smears were prepared from the thickest portion of each of the sputum samples. One set was then stained using the standard Ziehl-Neelsen method (*5*) and the other with the cold staining method. The 400 samples of sputum in the second group were concentrated using Petroff's method (*5*) and two smears were prepared from each of the concentrates. One set was then stained using the standard method and the other using the cold method described below.

Solutions for the cold staining method

Ziehl-Neelsen basic fuchsin-phenol solution, prepared as described by Allen & Baker (*5*).

Gabbet's methylene blue (*2*) containing:

Methylene blue	1 g
Sulfuric acid (AR grade)	20 ml
Absolute alcohol	30 ml
Distilled water	50 ml

[1] Assistant Professor of Microbiology, Institute of Tuberculosis and Chest Diseases, Madras, India.

[2] Professor of Tuberculosis and Chest Diseases, Madras Medical College and Director, Institute of Tuberculosis and Chest Diseases, Chetpet, Madras-600 031, India. Requests for reprints should be sent to this author at the latter address.

[3] Assistant Professor, Government Tuberculosis Sanatorium, Tambaram, Madras, India.

Staining procedure

Smears were heat-fixed on microscope slides, flooded with basic fuchsin–phenol stain, and allowed to stand at room temperature for 10 minutes. The smears were then washed in running water, counter-stained with Gabbet's methylene blue for 2 minutes, and subsequently washed in tap water and air-dried.

All smears were then examined microscopically by experienced technicians using an oil-immersion objective. Smears were graded as positive if a minimum of 3 bacilli were observed after scanning the slide for 15 minutes, while slides on which no acid-fast bacilli were found at the end of this time were rechecked.

RESULTS

Of the 700 direct smears examined (Table 1), 229 were positive by the cold method, in contrast to 225 by the conventional method; statistically, there was no difference between the results of the two methods ($P>0.5$). A common group of 216 smears was positive in both methods. Of the 229 smears that were positive in the cold method, 13 were negative in the Ziehl-Neelsen method, while 9 of the 225 smears that were positive in the Ziehl-Neelsen method were negative in the cold method. These discrepancies probably arose because single slides were used for each direct sputum smear and the distribution of tubercle bacilli in the sputum samples was not uniform.

Similar results were obtained with the concentrated smears. Of the 400 concentrated sputum smears (Table 2), 170 were positive by the cold method and 171 by the standard Ziehl-Neelsen method. There was no difference statistically between the two methods ($P>0.5$). A group of 169 smears was positive in both the methods, and of the 170 smears that were positive in the cold method only one was negative in the Ziehl-Neelsen method. Furthermore, of the 171 smears that were positive in the Ziehl-Neelsen method only 2 were negative in the cold method, indicating almost 100% correlation between the two methods. There was no difference in the intensity of staining in both methods.

DISCUSSION

The major difficulty in staining tubercle bacilli arises because their surface is coated with an unsaponifiable waxy substance. For such bacteria, the success of any staining technique depends on the ability of the dye to uniformly penetrate the cell wall through this waxy barrier, while leaving intact the acid-fast character of the bacilli. In the conventional Ziehl-Neelsen method, this is achieved by heating the microscope slide during the staining process. This operation requires fairly precise control of the temperature of the slide and experienced operators to carry it out successfully. In developing countries such as India, where smear microscopy is the main tool used to identify and follow up cases of tuberculosis, most laboratories use spirit lamps as the principal source of heating. However, very often hot staining methods cannot be performed because either rectified spirit is not available or there is a shortage of suitably trained personnel.

Numerous attempts have been made to develop a cold staining procedure for acid-fast bacteria (*1*, *2*, *4*). In the method described here longer exposure favours the uniform penetration of the dye through the cell wall. The concentration of staining solution is the same as that used in the classical Ziehl-Neelsen method, and hence no extra cost is involved.

Use of a cold staining procedure has two distinct advantages: a high level of technical skill is not

Table 1. Comparison of the results of staining sputum (direct smears) from tuberculosis patients by the Ziehl-Neelsen method and the cold staining method

	Ziehl-Neelsen		
Cold staining	Positive	Negative	Total
Positive	216	13	229
Negative	9	462	471
Total	225	475	700

Table 2. Comparison of the results of staining sputum (concentrated smears) from tuberculosis patients by the Ziehl-Neelsen method and the cold staining method

	Ziehl-Neelsen		
Cold staining	Positive	Negative	Total
Positive	169	1	170
Negative	2	228	230
Total	171	229	400

required, thus simplifying the training of personnel; and the problem of procuring rectified spirit for spirit lamps is removed, making it suitable for use even in remote areas.

Heat fixation of smears on slides can be carried out using any available source of dry heat, such as a hot plate or even the closed lid of a boiling sterilizer. In addition, ready-made staining solutions are commercially available.

Although the cold method described here is slightly longer to perform than the conventional Ziehl-Neelsen technique, its low cost, ease of use, and efficacy make it universally applicable, especially in those laboratories where facilities are poor.

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RÉSUMÉ

MÉTHODE DE COLORATION À FROID DES BACILLES ACIDO-RÉSISTANTS

La méthode de Ziehl-Neelsen est probablement la technique la mieux connue et la plus usitée pour colorer les bacilles tuberculeux. Ces derniers se colorent mal en raison de la substance cireuse insaponifiable qui revêt leur surface et la coloration n'est possible que si le colorant peut pénétrer librement dans les bacilles à travers cette barrière superficielle. Dans la méthode de Ziehl-Neelsen, la perméabilité de la paroi bactérienne est augmentée par le traitement à chaud par un colorant basique constitué de fuchsine phéniquée. Pour obtenir de bons résultats avec cette méthode un contrôle assez précis de la température est nécessaire et l'opérateur doit être un technicien qualifié. Dans les pays en développement où la bacilloscopie est le principal moyen de diagnostiquer la tuberculose, la plupart des laboratoires utilisent des lampes à alcool comme source de chaleur. Toutefois, il arrive souvent que la méthode de Ziehl-Neelsen ne puisse être appliquée faute de personnel qualifié et d'alcool rectifié.

Le présent article indique qu'une variante de la méthode de Ziehl-Neelsen, pratiquée avec les mêmes réactifs, mais avec une période de coloration plus longue, peut être utilisée pour colorer les bacilles tuberculeux à froid. Dans cette nouvelle méthode, la fuchsine phéniquée basique est laissée en contact avec l'étalement pendant 10 minutes à froid, au lieu de 5 minutes avec chauffage intermittent comme dans la technique de Ziehl-Neelsen classique. Un groupe de 1100 étalements d'expectorations provenant de malades atteints de tuberculose pulmonaire ont été colorés par la nouvelle méthode et les résultats obtenus ont été comparés à ceux que fournissait la méthode classique: dans le cas d'étalements directs, la corrélation était d'environ 97%, alors que pour les étalements concentrés la corrélation dépassait 99%. Aucun chauffage n'étant nécessaire, la technique est d'exécution simple et peut être confiée à des opérateurs n'ayant reçu qu'une brève formation. En raison de son faible coût, de sa simplicité et de son efficacité, cette méthode est aisément applicable, en particulier là où les services de laboratoires sont réduits.

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